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Wholly Printed Polypyrrole Nanoparticle-based Biosensors on Flexible Substrate

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Printing has been widely used in the sensor industry for its speed, low cost and production scalability. In this work we present a wholly-printed polypyrrole (PPy) based biosensor produced by inkjet printing bioinks composed of dispersions of PPy nanoparticles and enzymes onto screen-printed carbon electrodes. Two enzymes, horseradish peroxidase (HRP) or glucose oxidase (GoD) were incorporated into the PPy nanoparticle dispersions to impart biosensing functionality and selectivity into the conducting polymer ink. Further functionality was also introduced by deposition of a permselective ethyl cellulose (EC) membrane using inkjet printing. Cyclic voltammetry (CV) and chrono-amperometry were used to characterize the response of the PPy biosensors to H_2O_2 and glucose. Results demonstrated the possibility of PPy based biosensor fabrication using the rapid and low cost technique of inkjet printing. The detection range of H_2O_2 was found to be 10 μM - 10 mM and for glucose was 1 - 5 mM.

1. Introduction

Trends including aging populations in western countries, increasing environmental legislation, the rise of genetic technology, and the emergence of ubiquitous computing have become growth drivers for the development of cheap and portable sensor applications [1-5]. The increasing demand for low cost, mass-producible sensor products promises a vast and rapidly evolving market for the sensor manufacturer. Printed chemical- and bio-sensors represent a distinct opportunity in specific areas such as point-of-care medical diagnostics and smart packaging [6-8]. Among all the printing techniques, inkjet printing shows great potential for the production of customised sensor interfaces due to its speed, low cost and suitability towards automation [9-12]. Inkjet printing is a non-contact and non-impact printing technique that provides a "drop on demand" deposition system that can transfer designed patterns onto conductive and non-conductive substrates. These unique deposition advantages makes the inkjet printing technique an important fabrication tool for novel commercial sensor production in the future [13].

Conducting polymers have been previously utilized as electrode materials in biosensing applications. Electrons can readily transfer between the conducting polymers and biomolecules, such as hydrogen peroxide (H_2O_2) and glucose [14, 15], during a redox process, producing a detectable electrocatalytic response which can be monitored by simple electrochemical methods such as cyclic voltammetry (CV) and amperometry. Polypyrrole (PPy) was amongst the first reported conducting polymers to be utilized in biosensing. Wollenberger et al. electrodeposited PPy with horseradish peroxidase (HRP) onto pyrographite or platinum electrodes for mediatorless hydrogen peroxide detection[16]. Since then, a series of conducting polymer biosensors have been studied for the detection of various bio-molecules, including glucose, lactate and cholesterol [17, 18]. Although electrochemically fabricated conducting polymer/enzyme biosensors have been extensively studied, these fabrication methods are still limited to a laboratory scale and are not suitable for mass production.

Recently, we have successfully synthesized inkjet printable PPy nanoparticles and shown their potential application as a platform for nerve cell stimulation in tissue engineering [19, 20]. In this work, we utilized this printable PPy formulation to fabricate a wholly printable electrochemical sensor chip by introducing the enzymes HRP and glucose oxidase (GoD) into the ink to impart sensing functionality and selectivity. Bioinks of HRP and GoD blended with PPy formulations were prepared and the resultant inks were inkjet printed onto screen printed carbon electrodes (SPCEs) [21] to obtain PPy based biosensors. An ethyl cellulose (EC) membrane was then also jetted over the sensing areas to encapsulate them to prevent the enzyme leaching out from the PPy films (Fig. 1). This approach demonstrated a low-cost route to mass producing devices for commercially relevant sensing applications.

2. Experimental

2.1 Materials

Pyrrole, purchased from Sigma-Aldrich, was distilled and stored at –12 ˚C prior to use. Iron (III) chloride ($FeCl_3$) and PBS tablets were purchased from Fluka and used as received. Horseradish

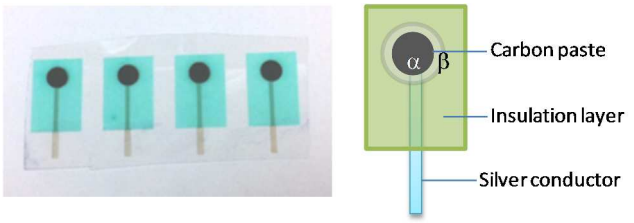

Fig. 1 (a) Strip of four bare SPCEs on Polyethylene terephthalate (PET); (b) Schematic of the inkjet printed PPy/enzyme biosensor based on an SPCE. (α) inner black circle:PPy/enzyme film on carbon paste (diameter: 5.5 mm); (β) outer white circle covering inner black circle: EC membrane covering PPy/enzyme electrode (diameter: 7 mm).

peroxidase (HRP, 250-330 units/mg), glucose oxidase (GoD, Type X-S, 100-250 units/mg), iron(III) *p*-toluenesulfonate hexahydrate (FepTS), polyvinyl alcohol (MW 31K-50K) (PVA), ethyl cellulose (EC) and dialysis membrane (Mw 12,000) were from Sigma-Aldrich and used as received.

Screen printed carbon electrodes (SPCEs) fabricated on flexible polyethylene terephthalate (PET) substrates were produced at the National Centre for Sensor Research, Dublin City University, via a previously reported method [20]. Silver ink (Electrodag PF-410) acting as a conductive layer was printed by DEK Albany 247 printing machine and cured in an oven at 120°C for 5 min. Carbon past ink (No. C10903D14, Gwent Electronic Materials Ltd., UK) was applied and cured at a range of curing temperatures from room temperature to 200°C for 15 min. A nonconductive dielectric layer (Electrodag 452 SS BLUE) was applied and cured in a UV curing machine for 3 cycles to work as insulation layer preventing the potential influence of silver conductor on sensor electrochemical behavior. The carbon paste electrode area was defined to be 0.071 cm^2.

2.2 Synthesis of inkjet printable PPy formulation

Inkjet printable PPy formulations were synthesised following the methods reported in our previous published work [19]. Polymerization was carried out at 0 ℃. 0.1 M Pyrrole and 1.2 % PVA (MWt 31K-50K) were mixed thoroughly with using 0.00625M Gemini surfactant 9BA-4-9BA(6,6'-(butane-1,4-diylbis(oxy)) bis(3-nonylbenzenesulfonic acid)) under magnetic stirring for 30 minutes in 40 mL distilled water under an ice-water bath. 0.1M FepTS and 0.4M $FeCl_3$ oxidants were dissolved in 10 mL water and added into the monomer dispersion at a constant rate 0.5 mL/min. The reaction continued for 24h and then was stopped. Polymerized dispersions were purified by dialyzing against Milli-Q water using a 12,000 Mw cut-off dialysis membrane (Sigma) for 48 hours with the water being changed every 16 hours to remove byproducts and excessive oxidants and surfactants. PPy nanoformulations were used directly after dialysis.

2.3 Preparation of PPy/HRP formulation

2.5 mg of HRP was dissolved in 1 mL of PPy dispersion and shaken by a vortex mixer to make an inkjet printable formulation. The resulting PPy/HRP formulation was ultrosonicated for 20 min and filtered (0.45 μm) before use.

2.4 Preparation of PPy/GoD formulation

5 mg of GoD was dissolved in 1 mL of PPy dispersion and shaken by a vortex mixer to make an inkjet printable formulation. The PPy/GoD formulation was ultrosonicated for 20 min and filtered (0.45 μm) before use.

2.5 Fabrication of PPy/enzyme biosensors

PPy/enzyme biosensors were inkjet printed using a piezoelectric Dimatix Materials Printer 2800 (DMP 2800), equipped with a 10 pL cartridge (DMCLCP-11610). The inkjet printable PPy/enzyme formulation was printed onto the SPCEs at 25.0 V, a frequency of 5.0 kHz, using a customized waveform to generate single layer and five layers films. The printed pattern was designed using Microsoft Visio software with an inkjet drop spacing of 25 μm. The printed sensors were left in air at room temperature for 2 hours to dry before a 0.5% w/v ethyl cellulose (EC) in butanol solution was jetted over the PPy/enzyme surface to cover a larger area than the underlying deposit (Fig. 1). This single EC layer was deposited using a 10 pL cartridge at 20.0 V, 5.0 kHz, using the DMP standard waveform, at a drop spacing of 30μm.

2.6 Characterization of printed sensors

Film thickness and surface roughness were characterized by using a Veeco Wyko NT 9100 optical profilometer. Scanning electron microscopy (SEM) images were taken using a JSM-7500F A Field Emission SEM. The contact angles of PPy/enzyme ink as test liquids, on SPCEs were determined with an OCA20 (DataPhysics Instruments GmbH) optical contact angle measuring instrument.

The cyclic voltammograms (CV) and chrono-amperometry responses of the fabricated biosensors to PBS and H_2O_2 or glucose were observed using a CHI 900b electrochemical system. Inkjet printed PPy/enzyme films on SPCEs served as working electrodes with a platinum mesh as counter electrode and Ag/AgCl as reference electrode. All the tests were carried out in 0.01M PBS solution. The pH value of the PBS solution was 7.4. All characterization was carried out directly under stirring without the injection of nitrogen or oxygen.

3. Results and discussion

3.1 Ink properties

The properties of the PPy ink were characterized after ultrasonication and filtering. The contact angle on the bare SPCE was measured to be 57.4° using the PPy ink as a test liquid, less than 90°, indicating this substrate is hydrophilic to some extent and well defined patterns are possibly achieved through printing to generate a continuous film. The solution properties for the ink media were found to have a surface tension of 48.6 ± 0.5 x 10^2 mN/cm with a viscosity of 28.4 ± 0.2 mPa/s. The incorporation of enzyme into the PPy ink slightly increased the viscosity and surface tension of the ink formulation to 30.2 ± 0.2 mPa/s and 51.4 ± 0.5 mN/m respectively, which made the ink suitable for forming a uniform film coating on the SPCE using the DMP 2800 printer equipped with a 10 pL cartridge.

3.2 Morphologies of inkjet printed biosensors

The carbon paste on the SPCE was first characterized by SEM before depositing the sensor material. SEM demonstrated that the screen printed carbon paste was quite rough at the micrometre

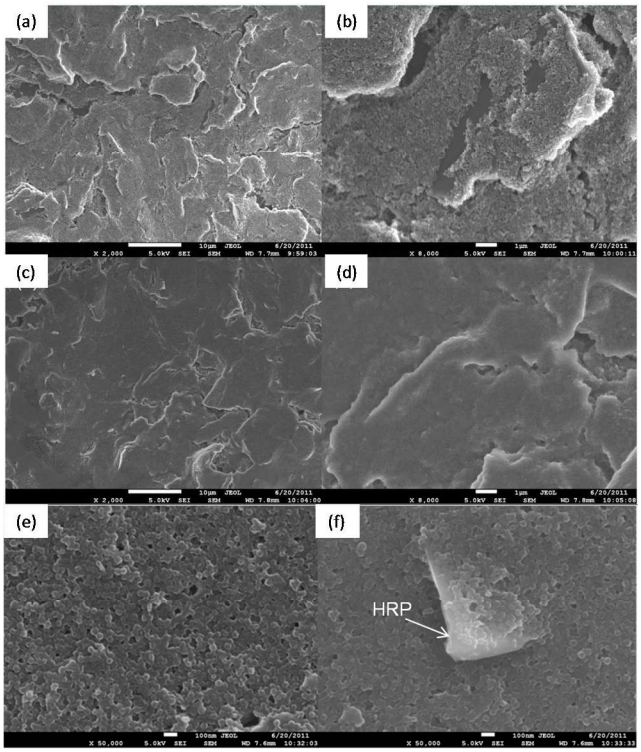

Fig. 2 (a) SEM image of bare SPCE, scale bar: 10 μm; (b) SEM image of bare SPCE, scale bar 1 μm; (c) SEM image of one layer of PPy film on SPCE, scale bar, 10 μm; (d) SEM image of one layer of PPy film on SPCE, scale bar, 1 μm; (e) PPy nanoparticles that constituted 1 layer PPy/HRP film, scale bar: 100 nm; (f) Detail of PPy particles encapsulating HRP enzyme crystal, scale bar 100 nm.

scale (Fig. 2 (a and b)). The carbon paste comprises small connected or overlapping carbon sheets instead of a continuous and uniform film. Randomly distributed gaps of around 0.5 μm can be observed over the entire carbon paste electrode surface. An underlying silver layer is used in the SPCE as shown in Fig. 1, and may influence the electrochemical performance of the biosensors due to pinholes in the carbon layer, as silver is electrochemically unstable. To solve this problem, a single layer enzyme-free PPy film was pre-printed on the SPCE to fill any gaps in the carbon layer, reducing surface roughness and preventing contact between the PPy/enzyme layer and silver electrode. It was found that most of the carbon sheets were covered by this single PPy film following inkjet printing and the carbon paste morphology became much smoother and more uniform (Fig. 2 (c and d)). The reduction in roughness was characterized by profilometry on an area of 47 x 62 μm (Fig. 3). The average roughness (Ra) decreased dramatically from 397.06 nm to 270.45 nm after the PPy layer was deposited.

The underlying roughness of the SPCE influences the observed roughness of the deposited PPy ink layer. For this reason, rather than trying to make accurate measurements on the irregular SPCE, surface thickness of the deposited PPy/enzyme layers was estimated by printing the PPy/enzyme ink onto glass slides and determining thickness by optical profilometry. Results indicated an average thickness of the single printed PPy/enzyme layer of approximately 70 nm. High resolution SEM image of the film surface (Fig 2 (e)) shows that the size of the PPy nanoparticles is around 50 nm. The film was continuous at the micrometre scale

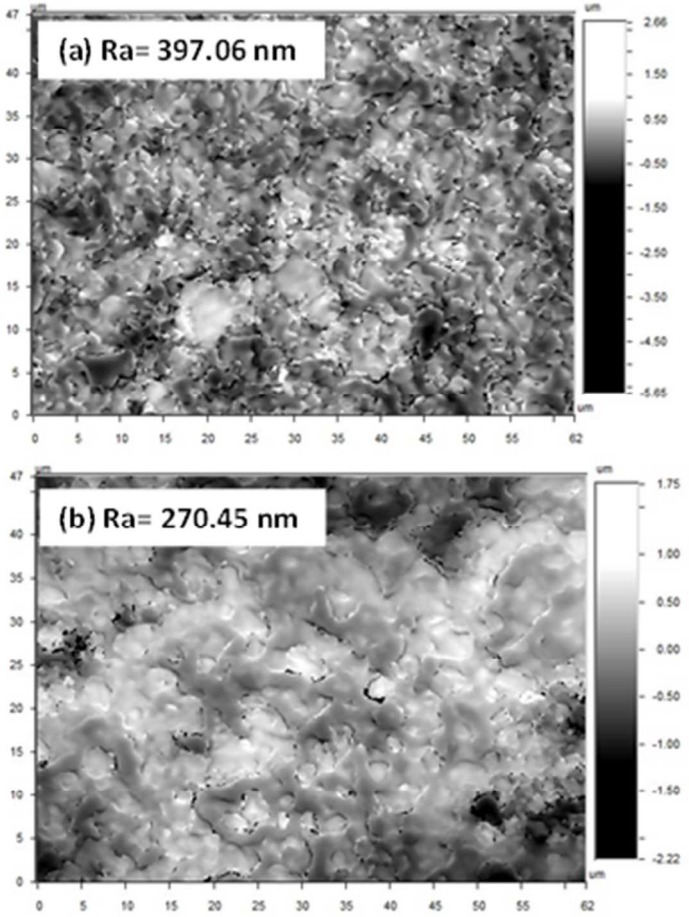

Fig. 3 Profilometry images and average roughness of (a) Bare SPCE; (b) single layer PPy film on SPCE.

and no obvious gaps could be seen on the film surface. Due to the high roughness of the underlying SPCE substrate, the detailed surface morphology was not as smooth and uniform as that printed on glass slides[20]. However, this specific morphology was actually beneficial to biosensor applications as the rough nanostructures can offer larger surface areas of the sensor electrode and was assumed to improve the charge transfer efficiency. Figure 2(f) demonstrates the presence of the enzyme (HRP) in the PPy films. It was noted that HRP crystals with a size of around 0.5 μm were embedded and stabilized in the PPy nanoparticles. This enhanced the contact area and connection between the enzyme active sites and the PPy host compared to other chemical and electrochemical fabrication methods [22, 23]. To prevent the water-soluble enzyme crystals from leaching into the PBS solution during sensor characterization, a single layer of EC membrane was printed from a 0.5% EC butanol solution as encapsulation layer (Fig. 1 (b)). These thin EC films were noted to form permeable membranes that allow small molecules including glucose and hydrogen peroxide to pass through, but were virtually insoluble in water; thereby preventing enzyme molecules from leaching. The thickness of this EC layer was also characterized with single layer jetted film on glass slide using optical profilometer and was determined to be around 30nm.

3.3 Electrochemical characterization of PPy based biosensors

CV of the printed PPy/HRP was carried out to determine whether

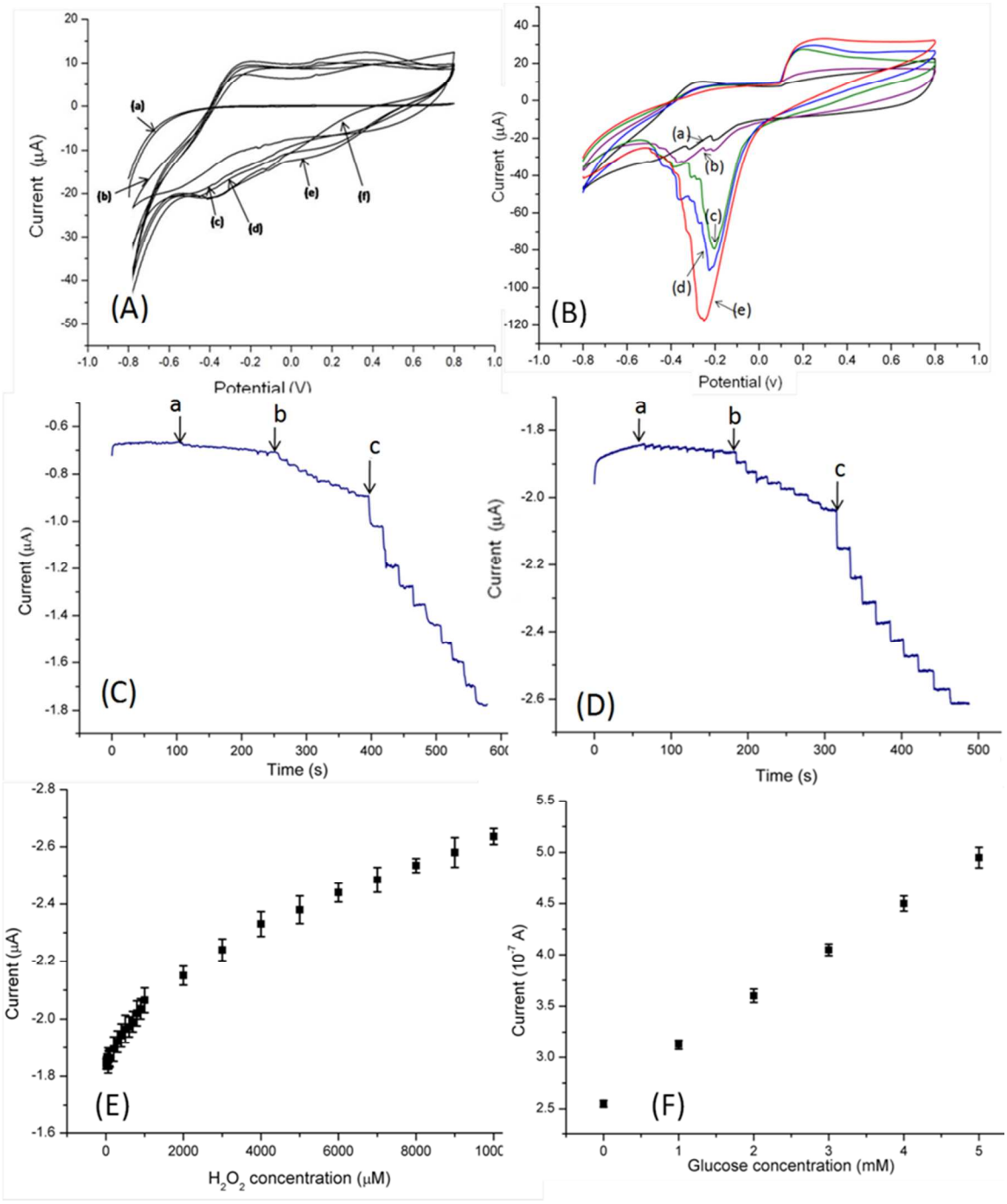

Fig. 4 (A) CVs of bare and modified SPCEs in 0.01 M PBS with H_2O_2 concentrations as stated. The potentials are vs. Ag/AgCl (3.0 M NaCl). Scan rate: 0.1 V/s (a) Bare SPCE; (b to f) Single layer PPy film in 0, 1, 10, 100 and 1,000 μM H_2O_2, respectively; (B) CVs of single layer inkjet printed PPy/HRP film in 0.01 M PBS, (a-e) 0, 1, 10, 100 and 1,000 μM H_2O_2, respectively. HRP loading is 2.5mg/mL. The potentials are vs. Ag/AgCl (3.0 M NaCl). Scan rate: 0.1 V/s;Chronoamperograms of (C) single layer inkjet printed PPy/HRP film and (D) five layers inkjet printed PPy/HRP film. Applied potential: -0.2 V. HRP loading: 2.5mg/mL. a-c are the starting points of the addition of 10^{-5} 10^{-4} and 10^{-3}M H_2O_2; (E) The calibration curve of the five layers PPy/HRP biosensor to H_2O_2 from 10^{-5} to 10^{-2} M; (F) The calibration curve of a single layer PPy/GoD biosensor to glucose from 1 to 5 mM. Error bars represent RSD of five measurements.

the HRP enzyme was compatible with the PPy formulation. The direct reduction process of H_2O_2 can be described using the formula below:

$$H_2O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad (1)$$

In the presence of HRP, H_2O_2 combines with HRP to form a HRP compound, followed by the reduction of this compound to the original HRP. This reduction needs lower energy than direct reduction of H_2O_2, thus was expected to occur at a higher potential.

$$\text{HRP (Red)} + 2H_2O_2 \rightarrow \text{HRP compound (Ox)} + 2\,H_2O \quad (2)$$

$$\text{HRP compound (Ox)} + 2e^- + 2H^+ \rightarrow \text{HRP (Red)} + H_2O \quad (3)$$

Experimental results agreed well with the assumption discussed

above (Fig. 4). The CV of a single layer PPy film changed slightly with the addition of H_2O_2 (Fig. 4 (A)). A small reduction peak was observed at -0.42 V vs. Ag/AgCl while oxidation current was nearly unchanged. The response current of this reduction peak increased with the concentration of the H_2O_2 but peak current was very low, indicating that PPy was not sensitive to the change of H_2O_2 concentration. Fig. 4 (B) illustrates that incorporation of the HRP enzyme into the PPy film successfully introduced a more significant H_2O_2 response. The potential of the H_2O_2 reduction peak increased to -0.25 V vs. Ag/AgCl, indicating that reduction in the presence of HRP proceeded more efficiently than direct reduction of H_2O_2. In addition, the reduction peak current at -0.25 V of the single layer PPy/HRP film increased significantly with the concentration of H_2O_2 compared to a single layer PPy film, illustrating that the sensitivity of the PPy/HRP films to H_2O_2 was much higher than PPy films alone.

From the above CV results, a constant potential of -0.2 V vs. Ag/AgCl, corresponding to the peak potential of the HRP-H_2O_2 reduction response, was applied to the PPy/HRP biosensors to monitor the amperometric detection of H_2O_2. Amperometric detection was carried out at pH 7.4 as this was close to the standard physiological environmental pH. Amperometry of a single layer and five layers of printed PPy/HRP films (HRP 2.5 mg/mL) were characterized by successively adding H_2O_2 (from 10^{-5} M to 10^{-2} M) to 0.01 M PBS solutions, under stirring, to determine the influence of PPy and HRP loading on the selectivity and sensitivity of the biosensor. The results showed that both single layer and five layers printed PPy/HRP films gave clear responses to the successive addition of H_2O_2 from 10^{-5} M to 10^{-2} M (Fig. 4 (C and D)). The response time for both sensors was 3 s, indicating that the response time was not affected by film thickness within this range (70 nm – 350 nm).

A H_2O_2 amperometric response calibration curve, of an inkjet printed five layers (~350 nm thick) PPy/HRP biosensor is shown in Figure 4 (E). The sensor exhibited a curve but had a nearly linear response over a broad range of H_2O_2 concentrations from 10^{-5} M to 10^{-2} M. The repeatability of the sensor was characterized by employing one sensor to record five H_2O_2 calibration curves in 0.01 M PBS solution in one day. The biosensor was rinsed with the 0.01M PBS solution between each measurement. The concentration value ranges from 10^{-5} M to 10^{-2} M for each measurement. Results showed that the relative standard deviation (RSD) of the slope in this concentration range was always below 3% (n=5) (Fig. 4 (E)). The calibration equation of the response current over this linear range was y=-7.543x - 1.941 (r^2 = 0.937). The detection sensitivity of the five layered PPy/HRP sensor to H_2O_2 was around 1.42±0.05 μA mM^{-1} cm^{-2}, which is much higher than layered printed PEDOT/HRP hydrogen peroxide sensor (0.544 μA mM^{-1} cm^{-2})[24].

A control experiment was carried out to make comparison between two fabrication methods: mixing enzyme directly into the ink formulation or printing enzyme covering conducting polymer layer. The later one was done following Setti's method[24]. 10 layers PPy film was firstly deposited on SPCE and then followed by printing 1 layer 2.5mg/mL HRP film over PPy film. Finally a layer of EC membrane was deposited as encapsulation layer as used in mixed PPy/enzyme method. PPy/HRP sensor obtained using this method only shows sensitivity around 0.25

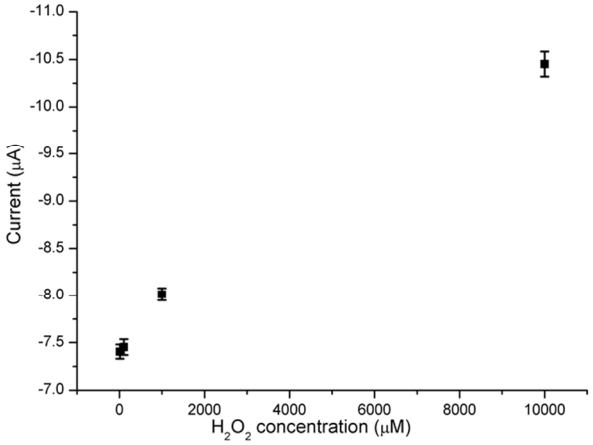

Fig. 5 The reproducibility calibration curve of five single layer inkjet printed PPy/HRP biosensors. Error bars represent RSD of single measurement of five sensors.

μA $mM^{-1}cm^{-2}$, illustrating that charge transfer between conducting polymer and enzyme is better when mixed together in comparison with simply printed layer by layer.

The single layer (70 nm thick) PPy/HRP biosensor had a similar calibration response with slightly lower detection sensitivity (0.96 μA mM^{-1} cm^{-2}) compared to the five layers sensor. The improved sensitivity of the five layers sensor was due to the increased amount of HRP. However, the sensor sensitivity did not increase proportionately with the number of printed layers. This was a result of the previously deposited HRP being buried within the proceeding printed layers thus limiting diffusion of the H_2O_2 to the HRP. The thinner film showed higher efficiency in H_2O_2 detection. Therefore, the single layer configuration was studied in the PPy/GoD sensor for glucose sensing.

To characterize the reproducibility of PPy/HRP biosensors, five single layer sensors in the same row on substrate sheet were printed at the same time and used to record H_2O_2 calibration curves in 0.01 M PBS solution in one day. 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M H_2O_2 were added successively into PBS solution for reproducibility characterization. Results showed that RSD of the current response at every concentration were all lower than 1.5% (Fig. 5), demonstrating inkjet printed PPy/HRP biosensors possessed highly reliable reproducibility. That's due to the great reproducibility of inkjet printing technique. The accurate same amount of materials was printed on the substrate and the amount of materials deposited can be tuned easily and effectively.

The stability of the single layer PPy/HRP biosensor was studied by recording the amperometric response to H_2O_2 under standard test conditions over time. Biosensors were stored in vacuum dryer under refrigeration at 4°C between measurements. Measurements were performed after 2, 6, 12, and 30 days storage. Results showed that 95.6%, 93.2%, 92.6%, 91.8%, 90.5% of the initial sensitivity was obtained, respectively. This biosensor retained more than 90% of the original sensitivity after 30 days storage, which was a significant improvement when compared to other similar devices described in the literature; such as an HRP entrapped carbon paste sensor which showed a 70% signal decrease after 1 day of use [25]. This stability can be attributed to the presence of PPy nanoparticles and the EC membrane which

minimised the loss of HRP enzyme through leaching from the biosensor film during characterization and the protection offered by PPy nanoparticles against deactivation of the HRP [26].

The possibility of extending the PPy/enzyme sensing system to other enzymes and biomolecules was also investigated. A PPy/GoD glucose sensor was fabricated using the same method as for the PPy/HRP sensors. The loading of GoD was 5 mg/mL. Amperometric detection was carried out to characterize the electrochemical behaviour of a single layer inkjet printed glucose biosensor (Fig. S3). The average response time calculated from three measurements was 4.3s, slightly higher than that of the PPy/HRP sensor. A linear response was observed from 1 to 5 mM, showing that inkjet printed PPy/GoD sensor worked effectively for glucose detection (Fig. 4(F)). Repeatability of PPy/GoD glucose sensor was measured using the same method as PPy/HRP H_2O_2 sensor. A RSD value which is below 2% was observed from test results, which is also similar to PPy/HRP sensor. The linear calibration equation of the response current was y=0.471x +2.603 (r^2 = 0.997). The sensitivity of this biosensor was 0.21±0.005 μA mM^{-1} cm^{-2}, which was not as high as the PPy/HRP biosensors. This may be due to the comparatively low activity of GoD as compared to HRP. The long term stability was also not as good as the PPy/HRP biosensor; with 85.6% of the initial sensitivity retained after one week of storage in vacuum dryer under refrigeration at 4°C, and by day 30 was 71.2% of the original value. Further studies will be carried out to investigate alternative methods to retain higher activity of GoD in the PPy formulation and to improve the storage protocol to minimise any loss of sensitivity.

Conclusions

Printed PPy/enzyme biosensors were successfully fabricated by inkjet printing PPy nanoparticle/enzyme bioinks onto flexible screen printed carbon electrodes. The incorporation of various enzymes introduced selectivity and sensitivity to specific biomolecules, such as H_2O_2 and glucose. Printed biosensors gave stable and continuous responses to H_2O_2 or glucose over a relatively broad range (H_2O_2: 10^{-5} to 10^{-2} M, glucose: 1 to 5 mM). Sensor sensitivity varied with the loading of the materials. Studies on repeatability and reproducibility revealed that these inkjet printed sensors had great reproducibility and were highly reliable in repeated measurements. Long term stability studies indicated that PPy/HRP biosensors retained more than 90% of initial sensitivity after 30 days vacuum dry storage at 4 °C under refrigeration.The stability of PPy/GoD sensors (~ 70% retention) was not as good as PPy/HRP sensors, and further studies on the protection of the GoD enzyme in the PPy formulation and under storage will be carried out in an attempt to address these issues.

Notes and references

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